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Human receptor Smoothened, a mediator of Hedgehog signalling, expressed in its native conformation in yeast☆

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Abstract Though the role of Hedgehog (Hh) signalling in patterning and differentiation during development is well established, the underlying signal transduction mechanisms remain obscure. This is the first report on the overexpression of the human Hh signalling receptor Smoothened (hSmo) in *Saccharomyces cerevisiae* and *Pichia pastoris*. We show that hSmo is expressed in both types of yeast in its native conformational state. The first purification presented here will allow the characterisation of hSmo expressed in yeast, and the scale-up of hSmo production enabling structural studies to develop new therapeutic approaches against tumors and neurodegenerative diseases induced by Hh signalling dysfunction.

Keywords: Heterologous expression; Membrane protein; Yeast; Smoothened; Hedgehog signalling; Purification

1. Introduction

Signalling proteins of the Hedgehog (Hh) family are essential for patterning and morphogenesis in most multicellular organisms [1]. Cellular responses to the secreted Hh are mediated by two integral membrane proteins, Patched (Ptc) and Smoothened (Smo), which were first identified by genetic screens in *Drosophila* [2,3]. Activation of the Hh pathway is triggered by binding of the Hh protein to its receptor Ptc [3,4], thereby alleviating Ptc-mediated suppression of Smo, a seven-transmembrane protein of the G protein coupled receptors family [5]. Smo activation then triggers a series of intracellular events that result in the expression of Hh target genes through the Ci/Gli family of transcription factors [6]. Despite findings suggesting that Ptc controls Smo function by influencing its interactions with small cellular molecules [7,8], and that Smo directly interacts with a member of the cytoplasmic signalling complex [9,10], the core mechanism of Smo's action remains essentially unknown [11–13]. Moreover, recent studies suggest that dysfunction of the Hh pathway in stem or precursor cells might contribute to tumorigenenesis and neurodegenerative disorders, making the components of this pathway prime targets for anticancer and antineurodegenerative therapies [14–16].

Knowing the structure of Hh receptor is essential to understand how this protein functions and how its activity can be modified by small molecules, and to elaborate new molecules able to inhibit or activate it. However, Smo, like the majority of medically important mammalian membrane proteins, is present in tissues at very low concentrations, making overexpression in heterologous systems a prerequisite for structural studies [17,18].

We show in the present paper that the *Human* receptor Smo can be expressed in its native conformational state in yeast *Saccharomyces cerevisiae* and *Pichia pastoris*, and purified.

2. Materials and methods

2.1. Construction of expression vectors

For *S. cerevisiae*, we used the YEpGAL vector containing the GAL1-10 promotor and the YEpPMA vector containing the plasma membrane proton ATPase (PMA) promotor, generously given by Al-Shawi and co-workers [19]. For *P. pastoris*, we used the pAO815 vector containing the Alcohol oxidase (AOX1) promoter/terminator cassette (Invitrogen). The Multitag Affinity Purification (MAP) sequence encoding (i) a factor Xa, a TEV and a thrombin cleavage sites to eliminate the MAP sequence; (ii) a calmodulin binding domain (CBD), a streptavidin tag and an hexahistidine tag for affinity chromatography; and (iii) an hemagglutinin peptide (HA) for anti-HA Western-blot analysis (Ruel and Thérond, unpublished), was inserted into the *Bam*HI and *Xho*I restriction sites of the YEpGAL or YEpPMA polylinker, and into the pAO815 *Eco*RI restriction site, to yield YEpGAL-MAP, YEpPMA-MAP and pAO815-MAP vectors. PCR with the Proofstart polymerase (Qiagen) was carried out on the hSmo cDNA I.M.A.G.E. (Consortium CloneID 4127774 [20]) to introduce at

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Abbreviations: Hh, Hedgehog; hSmo, human Smoothened; Ptc, Patched; AOX1, alcohol oxidase 1; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether); CBD, calmodulin binding domain; HA, hemagglutinin A; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DDM, *n*-dodecyl-β-D-maltoside; CHS, cholesteryl hemisuccinate; CHAPS, 1-propanesulfonate; DDAO, dodecyldimethyl-*N*-amineoxide

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the 5′ end two restriction sites (*Xba*I, *Spe*I) and a sequence of six adenosines, and at the 3′end an *Nhe*I restriction site, using the following primers: 5′-ACT AGT TCT AGA GAG CTC CCG CGG AAA AAA ATG GCC, and 5′-GGT ACC TCT AGA TCA GCT AGG GAA GTC CGA GTC TGC. The PCR product was subcloned in the pCR™2.1 plasmid (Invitrogen) and sequenced. HSmo cDNA was then digested by *Spe*I and *Nhe*I and subcloned in MAP *Xba*I/*Nhe*I sites, giving PAO815-hSmo-MAP, YEpPMA-hSmo-MAP and YEpGALhSmo- MAP.

2.2. Yeast strains and media

We used the *P. pastoris* strain GS115 (his4; [21]) (Invitrogen) and the *S. cerevisiae* strain K699 (Mata, ura3, and leu 2-3) generously given by R. Arkowitz. *P. pastoris* culture media (RDB plate, Minimal Glycerol Medium (MGY) and Buffered Methanol-Complex Medium (BMMY)) are described in Pichia Expression Kit Manual (Invitrogen). *S. cerevisiae* was grown on minimal medium (MM) (0.67% yeast nitrogen base without amino acids, 0.1 mM adenine, 0.2 mM uracil and an amino acid mixture lacking leucine) supplemented with 2% D-glucose, 2% D-fructose or 2% D-galactose.

2.3. Yeast transformation and culture

P. pastoris was transformed with PAO815-hSmo-MAP or PAO815-MAP, and cultured 48 h in MGY at 30 °C and 220 rpm. Around 6 OD_{600}, cells were centrifuged for 5 min at $430 \times g$, re-suspended in BMMY medium at 1 OD_{600}, grown at 30 or 20 °C under 220 rpm, and harvested at different times after methanol induction. *S. cerevisiae* was transformed with YEpGAL-hSmo-MAP, YEpPMA-hSmo-MAP, YEpPMA-MAP or YEpGAL-MAP, and grown at 30 or 20 °C and 220 rpm on MM supplemented with 2% glucose with YEpPMA vectors, or 2% fructose and diluted to 0.2 OD_{600} in MM supplemented with 2% galactose with YEpGAL vectors, until 5 OD_{600}, and centrifuged for 10 min at $430 \times g$ and 4 °C.

2.4. Crude extract and membrane preparation

All steps were performed at 4 °C. Yeast cells were washed in cold water, re-suspended in disintegration buffer (50 mM sodium phosphate (pH 7.4), 1 mM EDTA, 5% glycerol, freshly added protease inhibitors cocktail (PIC, Roche), and 1 mM PMSF), and broken by vortexing 1 h with glass beads (425–600 μm, Sigma). Unbroken yeast were pelleted for 10 min at $430 \times g$, and crude extract was obtained from the supernatant. Membranes were prepared by centrifugation of crude extract for 1 h at $180\,000 \times g$.

2.5. Gel electrophoresis and Western blotting

Protein samples were separated on 10% SDS–PAGE and silver-stained or transferred to nitrocellulose using standard techniques. Blots were probed with mouse anti-HA or rabbit anti-Smo (Santa Cruz Biotechnology), and developed using an ECL kit (Amersham Biosciences).

2.6. Solubilisation

Eighty micrograms of membranes from *S. cerevisiae* and *P. pastoris* were solubilised in buffer A (50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 20% glycerol, PIC, and 1 mM PMSF) plus 1.0% detergent and 0.2% cholesteryl hemisuccinate (CHS, Sigma) 1 h at 4 °C under gentle agitation.

2.7. Purification

All purification steps were carried out at 4 °C. Ten milligrams of membranes were stirred for 45 min in 2 ml of buffer A plus 1% *n*-dodecyl-β-D-maltoside (DDM, Calbiochem) and 0.2% CHS. The suspension was then centrifuged for 1 h at $180\,000 \times g$. One-third of the supernatant was supplemented with 2 mM $CaCl_2$, 1/3 with 10 mM imidazole and the other 1/3 without addition, and batch loaded during 1 h onto 100 μl of calmodulin Sepharose (Amersham), Ni–NTA agarose (Qiagen) or streptavidin Sepharose (Amersham) pre-equilibrated. The resin was then centrifuged for 1 min at $400 \times g$, re-suspended in 1 ml of wash buffer (buffer A plus 0.1% DDM, 0.02% CHS, and 2 mM $CaCl_2$, 10 mM imidazole or nothing) and centrifuged. This washing step was repeated three times. The resin was then incubated for 10 min with 100 μl of elution buffer (buffer A plus 0.1% DDM, 0.02% CHS and 4 mM EGTA for calmodulin sepharose, 500 mM imidazole for Ni–NTA agarose or 2 mM biotin for streptavidin Sepharose), and the eluate was collected after centrifugation for 1 min at $400 \times g$. The elution step was also repeated three times.

2.8. Protein quantification

The proteins were quantified using Bio-Rad protein assay.

2.9. Fluorescence binding assays

For the flow cytometry analysis (FACS Scan; Becton Dickinson), hSmo-expressing or control yeast cells were incubated overnight at 30 °C with 50 nM of BODIPY–cyclopamine (generously given by P. Beachy [7]), collected by centrifugation, and re-suspended in PBS buffer plus 1% paraformaldehyde. For fluorescence variation measurements (spectrofluorimeter SAFAS FLX-Xenius), 200 μg of membrane fraction with or without hSmo were incubated in PBS buffer and different concentrations of BODIPY–cyclopamine (between 0 and 25 nM) 4 h at RT, centrifuged for 1 h at $25{,}000 \times g$ and re-suspended in 1 ml of PBS buffer. The fluorescence variations. $\Delta F/F$ were calculated for each concentration of cyclopamine (F: membrane fluorescence without cyclopamine, ΔF: fluorescence intensity difference between membranes incubated with cyclopamine and membranes incubated without cyclopamine). The BODIPY–cyclopamine standard curve was realised by measuring the fluorescence variations of different BODIPY–cyclopamine concentrations in 1 ml of membranes without hSmo.

3. Results and discussion

3.1. *hSmo can be expressed in* S. cerevisiae *and* P. pastoris

Yeast is the simplest eukaryotic cell that performs many of the post-translational modifications seen in higher eukaryotic cells. Moreover, it is easy to grow in large volumes in a short period of time [22]. *S. cerevisiae* has been successfully used to functionally express and purify mammalian membrane proteins such as the human P-glycoprotein [19], the rat vesicular monoamine transporter [23], and the rabbit SERCA1a Ca^{2+}-ATPase [24]. With its preference for respiratory growth, the methylotrophic yeast *P. pastoris* can be cultured at extremely high densities [25]. The introduction of heterologous genes at the AOX1 locus by homologous recombination results in very high levels of mRNA and protein expression upon methanol induction [26]. The use of *P. pastoris* as a heterologous expression system has been successful for several mammalian integral membrane proteins such as the ATP-binding cassette transporters [27] and the human dopamine receptor [28]. After modification by PCR to introduce restriction sites and a sequence of six adenosines just upstream the ATG initiation codon to optimise translation initiation and proteins expression [29], the hSmo cDNA was subcloned in the expression vectors at the 5′-end of the MAP sequence. This MAP sequence fused at the hSmo's C-terminal end provides several epitopes to follow hSmo expression, as well as opportunities for rapid purification under mild conditions using several affinity chromatography columns (Fig. 1). *S. cerevisiae* transformed with YEpGAL-hSmo-MAP, YEpPMA-hSmo-MAP, YEpPMAMAP or YEpGAL-MAP, and *P. pastoris* transformed with pAO815-hSmo-MAP were screened for expression of hSmo at the plasma membrane by Western blotting using antibodies directed against the HA peptides present in the MAP sequence (anti-HA) or the C-terminal part of Smo (anti-Smo). We observed that in *S. cerevisiae*, hSmo is more expressed at 20 °C than at 30 °C (data not shown), consistent with various other studies suggesting that folding and stability of membrane proteins are often favorably influenced by low

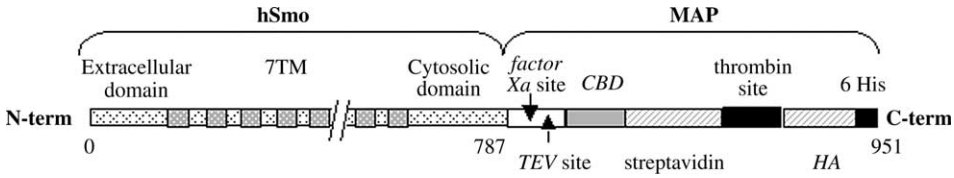

Fig. 1. MAP sequence fused at hSmo C-terminal end.

temperatures [23]. The hSmo expression decreases after 3 OD_{600} during the exponential growth, which has also been observed for the human adenosine receptor and explained as a result of translational or post-translational events [30]. Comparable expression levels were obtained under constitutive and inducible promoters, and 10% glycerol in growth medium did not significantly enhance hSmo expression, contrary to the human P-glycoprotein [19]. Fig. 2 shows that hSmo is expressed in *S. cerevisiae* membranes as a major protein migrating around 80 kDa. When higher quantities of membranes are loaded, we also observe a second band around 110 kDa (Fig. 4B). When grown at 30 °C, *P. pastoris* expressed maximal levels of hSmo in the membrane fraction 24 h after induction with methanol, and yielded a major signal around 80 kDa and a weaker signal around 110 kDa (Fig. 2). Lowering the growth temperature to 20 °C did not modify this expression pattern. The intensities of hSmo detected using anti-HA antibodies are comparable in *S. cerevisiae* and *P. pastoris* membranes (Fig. 2), suggesting that comparable amounts of hSmo per mg of membrane proteins are expressed in the two types of yeast. However, hSmo expressed in *S. cerevisiae* migrates slightly faster than hSmo expressed in *P. pastoris* and is less immuno-reactive to anti-Smo antibodies. This suggests that hSmo C-terminal domain may be slightly differently folded in *S. cerevisiae* in comparison to *P. pastoris*. The molecular weight calculated from the hSmo sequence is 104 kDa. The band at 80 kDa being the major product obtained in both types of yeast, we think that this apparent molecular weight results from atypical running behaviour of the whole non-glycosylated hSmo. This is frequently observed with membrane proteins due to their hydrophobicity [28,31], and does not result from proteolysis. When expressed in COS cells, the mouse Smo is glycosylated and migrates around 110 kDa on SDS–PAGE gel [7]. The 110 kDa product obtained in yeast could correspond to a glycosylated form of hSmo. Consistently with a non-glycosylated form of hSmo, deglycosylation treatment does not modify the 80 kDa product, but, due to its low expression level, we did not succeed in obtaining any significant result on the 110 kDa product (data not shown).

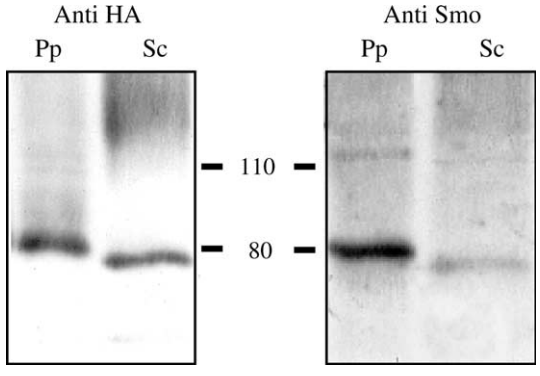

Fig. 2. hSmo expressed in *S. cerevisiae* (Sc) and *P. pastoris* (Pp) membranes. Western blot with anti-HA antibodies (left) and anti-Smo antibodies (right) on membrane proteins (20 μg) from YEpPMA-hSmo-MAP transformed *S. cerevisiae* grown at 20 °C until 2 OD, and from PAO815-hSmo-MAP transformed *P. pastoris* induced with methanol during 24 h at 30 °C.

3.2. *hSmo conformational state in* S. cerevisiae *and* P. pastoris

Chen et al. [7] showed that the steroidal alkaloid cyclopamine specifically binds to the mouse Smo heptahelical domain, and that this binding is very sensitive to the conformational state of Smo. As there are 87% of identity between mSmo and hSmo, we used a fluorescent derivative of this Smo antagonist (BODIPY–cyclopamine) to investigate the conformational state of hSmo expressed in yeast. After incubation with 50 nM of BODIPY–cyclopamine, hSmo-

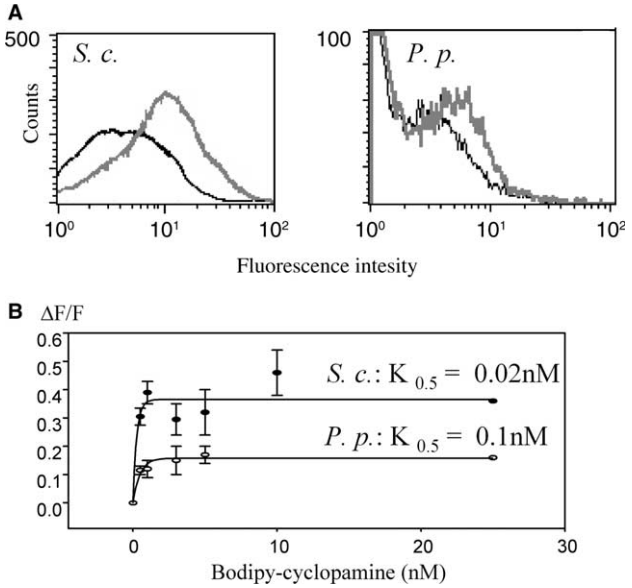

Fig. 3. Cyclopamine fluorescent derivative binding. (A) Flow cytometry analysis of yeast incubated with 50 nM BODIPY–cyclopamine overnight at 30 °C; left: YEpPMA-hSmo-MAP (grey line) and YEpPMA-MAP (black line) transformed *S. cerevisiae* grown at 20 °C until 2 OD_{600}, right: PAO815-hSmo-MAP (grey line) and PAO815-MAP (black line) transformed *P. pastoris* induced with methanol during 24 h at 30 °C. (B) Fluorescence variations of BODIPY–cyclopamine bound to 200 μg of membrane preparations from YEpPMA-hSmo-MAP transformed *S. cerevisiae* or PAO815-hSmo-MAP transformed *P. pastoris*. Experiments were repeated three times. F: fluorescence intensity in the absence of cyclopamine. Δ*F*: variation between fluorescence intensity at different concentrations of cyclopamine and fluorescence intensity without cyclopamine. Control experiments have been carried out with membranes prepared from *P. pastoris* or *S. cerevisiae* which do not express hSmo.

expressing *S. cerevisiae* and *P. pastoris* became, respectively, 4 and 2 times more fluorescent than control yeast transformed with empty vector. This indicates that at least part of hSmo expressed in both types of yeast is in a conformation allowing cyclopamine binding (Fig. 3). The fluorescence variations of *S. cerevisiae* and *P. pastoris* membranes as a function of the concentration of BODIPY–cyclopamine allowed to calculate a half-effect constant and a number of active hSmo per milligram of membrane proteins of 0.02 nM and 100 pmol for *S. cerevisiae*, and of 0.1 nM and 40 pmol for *P. pastoris*. We estimate that hSmo in a correct conformation to bind cyclopamine represents about 1% of membrane proteins in *S. cerevisiae* and 0.4% in *P. pastoris*, and that one liter of *S. cerevisiae* or *P. pastoris* culture would contain 300 or 800 μg of hSmo, respectively.

3.3. hSmo purification

S. cerevisiae and *P. pastoris* membranes were solubilised with 1% of different detergents: lubrol, DDM, CHAPS, dodecyl glucoside, DDAO and C12E8, which critical micellar concentrations are comprised between 0.004% and 0.1%, and analysed by Western blotting (Fig. 4). Since DDM is one of the most efficient detergents for hSmo solubilisation and one of the most frequently used non-ionic detergents for purification and crystallisation of membrane proteins, it has been chosen for hSmo purification. In order to test the efficiency of the different affinity resins for hSmo purification, *P. pastoris* membranes were solubilised with DDM in the presence of cholesteryl hemisuccinate (CHS). As described for other human GPCRs [28,31], addition of cholesterol derivative CHS to solubilisation experiments increases the stability of solubilised hSmo. Therefore all purification steps have been performed in the presence of CHS. Same amounts of solubilised hSmo have been incubated with Ni–NTA, calmodulin or streptavidin resin in adequate buffers, and the Western-blotting analysis of eluates indicates that Ni–NTA is the most efficient resin (Fig. 5). The eluate from Ni–NTA resin was loaded on SDS–PAGE gel for silver-staining. The three bands that appear, one at 80 kDa and two around 110 kDa, correspond to those observed on the Western blot presented in Fig. 5 and represent 40% of total proteins loaded on gel (Fig. 6).

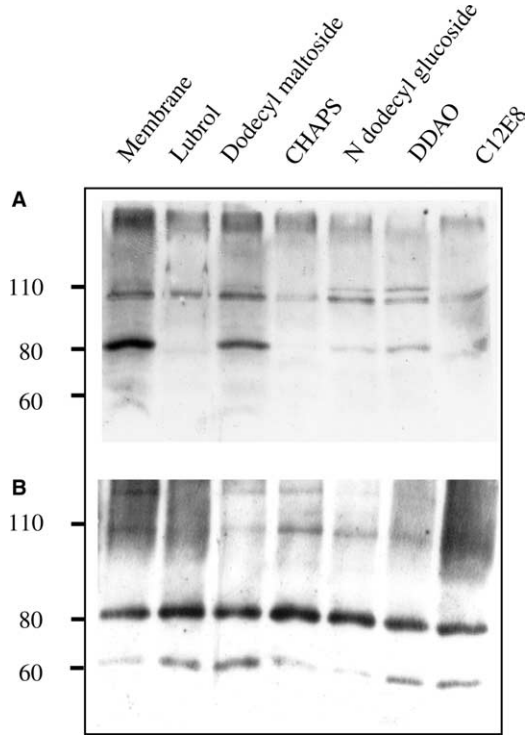

Fig. 4. hSmo solubilisation. 80 μg of membrane proteins prepared from PAO815-hSmo-MAP transformed *P. pastoris* (A) or YEpPMA-hSmo-MAP transformed *S. cerevisiae* (B) were solubilised with 1% of the different detergents, 1 h at 4 °C, loaded on 10% SDS–PAGE gel and immunoblotted with anti-Smo antibodies for *P. pastoris* and anti-HA antibodies for *S. cerevisiae*.

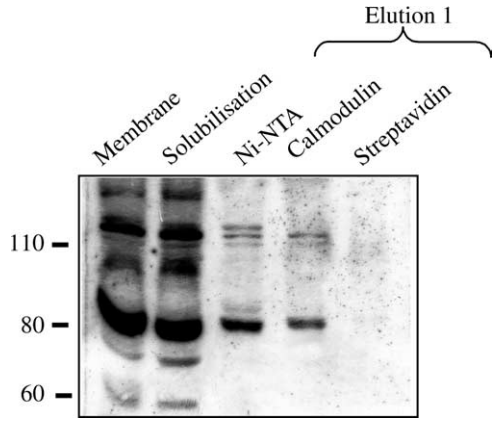

Fig. 5. HSmo purification. 2 mg of *P. pastoris* membranes were solubilised with 1% DDM in presence of 0.2% CHS and batch-loaded on 100 μl of each affinity resin: Ni–NTA, calmodulin and streptavidin, 2 h at 4 °C. Membrane, solubilised and eluates were loaded on 10% SDS–PAGE gel and immuno-detected with anti-Smo antibodies.

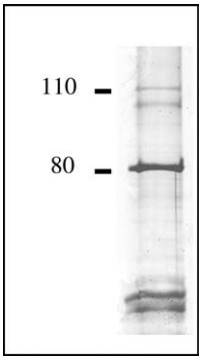

Fig. 6. Silver-stained SDS-gel of Ni–NTA eluate.

4. Conclusion

This study shows that the human Hh signalling pathway receptor Smo can be expressed in its native conformational state, in glycosylated and mainly non-glycosylated forms, in the membrane fraction of both types of yeast used as heterologous hosts: *S. cerevisiae* and *P. pastoris*. Smo is efficiently solubilised by dodecyl maltoside in the presence of cholesterol derivative, and the protein fraction obtained after Ni–NTA

resin purification contains 40% of hSmo. A second purification step using the calmodulin resin would allow the complete purification of hSmo. The expression levels reached in *P. pastoris* are high enough to undertake large-scale production of hSmo for structural studies.

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